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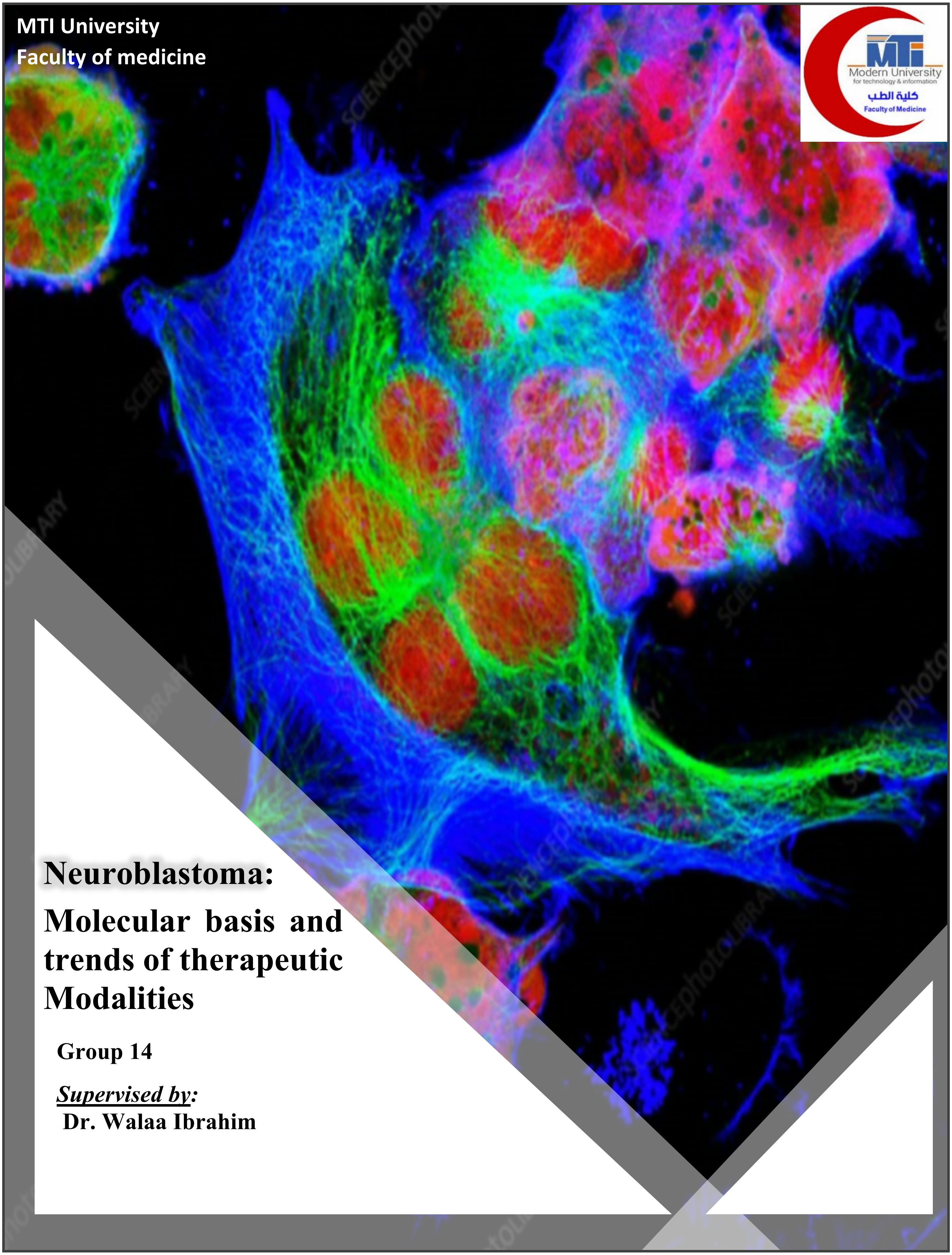

Neuroblastoma:
Molecular basis and trends of therapeutic Modalities

Group 14

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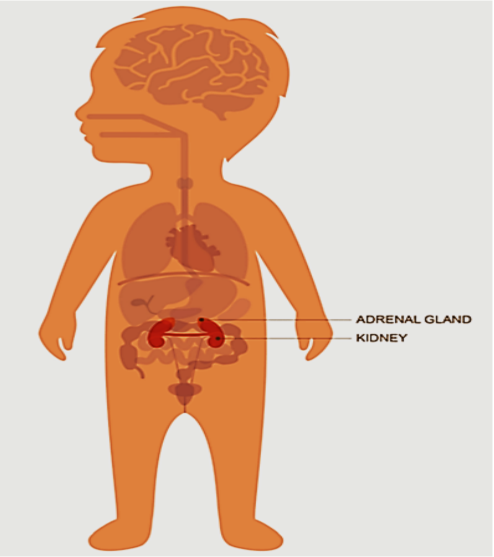
Abstract

Neuroblastoma is the most common extracranial solid tumor of infancy. It’s an embryonal malignant tumor of neural crest cells, that give rise to the sympathetic nervous system. It develops from immature nerve cells found in several areas of the body which mainly arises in and around the adrenal glands. There are two methods of neuroblastoma staging, one that is based on post-operative patients (INSS) (mainly for prognosis) and one developed for pre-treatment patients (INRGSS). Numerous chromosomal mutations have been reported in both localized and metastatic NB, with the structural mutations being more common in metastatic tumors like mutation in ALK gene and MYCN(a proto-oncogene which is expressed normally in tissues such as the kidney, spleen, lung and nervous system during embryonic development, is located on the short arm of chromosome), so MYCN amplification is used as molecular diagnostic approach technique as it's associated with advanced stage, rapid tumor progression, and poor prognosis. In addition to genetic mutations and SNPs, epigenetic factors may also contribute to disease, as any disturbances in (DNA methylation // and histone modification (hypoacetylation of histone 3 (H3))) may give rise to disorders, such as cancer. Finally, we discuss Recent therapeutic modalities of neuroblastoma such as :Immunotherapy strategies, Stem cell therapyand Epigenetics targeted therapies.

Background:

Neuroblastoma, an embryonal cancer of the autonomic nervous system, is the most common cancer diagnosed during the first year of life. Although neuroblastoma accounts for disproportionately high morbidity and mortality among childhood cancers, it has one of the highest rates of spontaneous and complete regression. It is a malignant tumour of neural crest cells, the cells that give rise to the sympathetic nervous system, which is observed in children. (1),(3)

It develops from immature nerve cells found in several areas of the body. Neuroblastoma most commonly arises in and around the adrenal glands, which have similar origins to nerve cells and sit atop the kidneys. However, neuroblastoma can also develop in other areas of the abdomen and in the chest, neck and near the spine, where groups of nerve cells exist. (2)



The incidence of Neuroblastoma is 10.2 cases per million children under 15 years of age, and nearly 500 new cases are reported annually. While 90% of cases are diagnosed before the age of 5, 30% of those are within the first year. The median age of diagnosis is 22 months. Rarely does it present in adolescence and adulthood, but outcomes are much poorer in this age group. There doesn't appear to be an increased prevalence among races, but there is a slight predilection for males (1.2:1). (3)

1. Stages of Neuroblastoma

There are two methods of neuroblastoma staging, one that is based on post-operative patients (INSS) and one developed for pre-treatment patients (INRGSS).

a. International Neuroblastoma Staging System (INSS)

This staging system is for post-operative patients and mainly for prognosis

- ***Stage 1***: localized tumor with complete gross excision with or without microscopic residual disease contralateral and representative ipsilateral regional lymph nodes negative for disease (nodes attached to and removed with primary tumor may be positive).
- ***Stage 2a***: localized tumor with incomplete gross excision ipsilateral and contralateral nodes negative for tumor.
- ***Stage 2b***: localized tumor with complete or incomplete resection positive ipsilateral (non-adherent) nodes contralateral nodes negative for tumor

- ***Stage 3*:** unresectable lateral tumor that crosses the midline or localized tumor with contralateral regional lymph node involvement midline tumor with bilateral extension by infiltration or by lymph node involvement.
- ***Stage 4S ("special")*** <1 year of age, localized tumor (stage 1, 2A or 2B) distant metastases confined to skin, liver and/or bone marrow.
- **Stage 4:** distant metastases not fulfilling stage 4S

In the majority of cases (60-80%), patients present with stage 3 or 4 tumors.

International Neuroblastoma Risk Group Staging System (INRGSS)

The INRG was developed after INSS to formulate staging for pre-operative neuroblastoma 5,6:

- ***Stage L1*:** localized tumors confined to one body cavity and not involving image-defined risk factors (IDRFs).
- ***Stage L2*:** locoregional tumors involving one or more IDRFs.
- ***Stage M*:** distant (remote) metastases (i.e. excludes metastases to local lymph node groups).
- ***Stage MS*:** metastases in patients <18 months (some centers <12 months) confined to skin, liver and/or bone marrow.

More than 20 image-defined risk factors (IDRF) have been extensively defined by the INRG 5,6, and these are outside the scope of this article. (4)

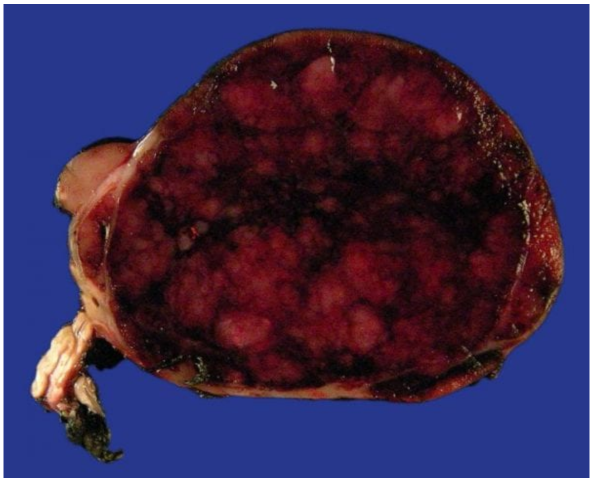
2. Pathological features

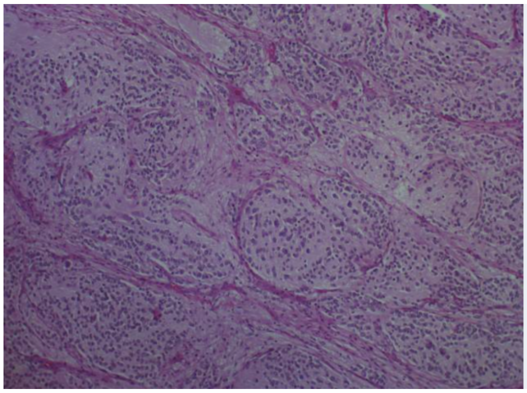
The origin of neuroblastoma cells from neural crest derivatives is generally accepted now, and this histogenesis explains some biochemical and morphological characteristics of the tumor. The cytological and histological features of neuroblastomas can be rather varying and, therefore, the diagnosis and differential diagnosis may be difficult. (5)

Gross

Localized tumor with:

- Variable size, circumscribed, ovoid mass to multilobated tumor.
- Hemorrhagic with vague, bulging lobules.





- ***Microscopic (histologic) picture***
- Small round blue cell tumor.
- Neuroblasts.
- Undifferentiated: small to medium, salt and pepper chromatin, elongated shape, may contain distinct nucleoli, indiscernible / small amount of cytoplasm, vague cytoplasmic borders.
- Differentiating (toward ganglion cells): synchronous differentiation of nucleus (enlarged, eccentric nucleus with vesicular chromatin and single prominent nucleolus) and abundant, eosinophilic / amphophilic cytoplasm.
- May have anaplastic, pleomorphic, spindled, rhabdoid variants.
- May form Homer-Wright pseudorosettes surrounding delicate, eosinophilic neuropil.
- Coagulation necrosis, fibrin, or collagen may be present.
- In poorly differentiated or differentiating subtypes, Schwann cells and differentiated / differentiating ganglion cells may be found (especially at tumor periphery). (6)

3. Immune escape in neuroblastoma

Cancer cells have evolved multiple mechanisms to escape immune recognition or to modulate immune cell function. Several subtypes of immune cells that infiltrate tumors can foster tumor development, harbor immunosuppressive activity, and decrease an efficacy of adoptive cell therapies. Therefore, an understanding of the dual role of the immune system under the influences of the tumor microenvironment (TME) has been crucial for the development of effective therapeutic strategies against solid cancers.(7)

Neuroblastoma tumors are lowly immunogenic due to their low mutational burden, which is associated with a low generation of neo-antigens . Tumor-associated antigens, which could be recognized by patrolling T cells, are therefore infrequent, although some neuroblastoma-associated immunogenic antigens have been identified . Also, the antigen-presenting machinery, consisting of MHC-I molecules, with which somatic cells constitutively present their intracellular antigens, is not functional in neuroblastoma . These features render neuroblastoma cells virtually invisible to patrolling $CD8^+$ T cells. Tumoural MHC-I expression in neuroblastoma impacts the immune response. MHC-I expression on tumor cells correlated directly with the density of tumor-infiltrating $CD3^+$ T cells, the latter being an important prognostic factor for survival.(8)

Due to their low MHC-I expression, neuroblastoma cells may not be susceptible to recognition by CTL, but should be rendered vulnerable to NK cells. *In vitro*, NK cells were indeed capable of killing neuroblastoma cells, although pre-activation of isolated NK cells was required in some settings.(7)

4. Different molecular biology techniques that could be used in molecular diagnostic approach of neuroblastoma

MYCN amplification values usually range between 50 and 100 fold, although much higher values have been reported. Since research has revealed the association of MYCN amplification with NB evolution , independent from disease stage and age at diagnosis, MYCN amplification has been used as the biomarker for risk stratification . Thus, assessment of MYCN amplification is essential for the diagnostic evaluation of patients with NB. MYCN amplification status in NB must be assessed across all conditions (i.e., new diagnosis, prognosis, prospective and retrospective) and in immediate facilities, without any limitations.

Since the original discovery of MYCN amplification in a substantial subset of patients with NB, a number of methodologies, including southern blotting , polymerase chain reaction (PCR), differential PCR , quantitative PCR (QPCR) , fluorescence in situ hybridization (FISH) /direct FISH , interphase quantitative FISH (IQ-FISH) , and chromogenic in situ FISH (CISH) have been validated as assessment methods. In addition, immunohistochemistry (IHC) can be a convenient and cost-effective approach.(9)

5. Genetic alterations in neuroblasts implicated in the development of neuroblasts

According to recent studies, age and genomic abnormalities are two predisposing factors in NB development. (10)

Numerous chromosomal mutations have been reported in both localized and metastatic NB, with the structural mutations being more common in metastatic tumors.

- **MYCN**

MYCN(V-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived [avian]), a proto-oncogene which is expressed normally in tissues such as the kidney, spleen, lung and nervous system during embryonic development, is located on the short arm of chromosome.(11)

MYCN amplification encoding the transcription factor N-MYC has been documented in most of the malignant neuroblastomas that cover about 20–25% of all NB .

MYCN amplification has also been associated with many chromosomal events, including loss of the distal short arm of 1p, aberration at 11q, and gain of 17q . N-MYC acts as a transcription factor recognizing a consensus sequence (CACGTG) and can activate genes that affect cell growth and differentiation . Thus, MYCN amplification is associated with advanced stage, rapid tumor progression, and poor prognosis.(9)

MYCN can also encode the v-Myc avian myelocytomatosis viral oncogene NB-derived homolog (N-Myc), another proto-oncogene with a proven effect on tumorigenesis in case of overexpression with MYCN amplification, especially in NB. These two genes also affect programmed death protein-1 (PD-1) regulation and can be used as good targets for NB treatment. (12)

ALK

ALK gene amplification is found to be related to NB and some other malignancies as a result of gene activating mutations. It regulates the mitogen-activated protein kinase (MAPK) pathway, which causes uncontrollable cell division by affecting growth factors. These mutations occur in 8–10% of patients with NB. (13)

This confirms that ALK is a suitable target for NB targeted therapy. Studies show that targeting ALK is an effective therapeutic strategy in NB therapy in patients either with either mutated or even normal ALK protein expression. (14)

GD2

GD2 is a disialoganglioside with minimum expression in some special tissues as the cerebellum and peripheral nerves while being over- expressed in cancers derived from the neuroendocrine system. Overexpression of GD2 in different tumor types has been observed in different patterns. GD2 antigen abundantly found on all NB cells due to their neuroectodermal origin and affects oncogenesis of NB through different pathways. GD2 serves invasion of NB by suppression of effector immune cells and phosphorylation of tyrosine kinases. (15)

6. Role of epigenetics in the development of neuroblastoma

In addition to genetic mutations and SNPs, epigenetic factors may also contribute to disease.

a. DNA methylation

DNA methylation at the fifth position of cytosine (5mC) in CpG dinucleotides is a major epigenetic mechanism involved in genome programming and reprogramming, and determination of cell fate during development, thus any disturbances in DNA methylation may give rise to disorders, such as cancer.(16)

Near gene promoters, transcription start sites, and/or first exons, CpG dinucleotides cluster in short DNA stretches, which are referred to as CpG islands (CGI), and most (80–95%) of them remain devoid of DNA methylation (17)The majority of the CpG sites occur in intergenic regions and repetitive genomic sequences to maintain a transcriptionally inactive state (18). Changes in the cancer epigenome are generally associated with loss of global DNA methylation and gain of DNA methylation at specific gene promoters. The consequences of loss of global methylation include chromosomal instability, loss of imprinting, and activation of transposable elements, thereby leading to disturbances in the genome (16) . Genome instability causes cancer genotypes to continuously change and evolve and can manifest itself genetically on several different levels, ranging from simple DNA sequence changes to structural and numerical abnormalities at the chromosomal level, all of which are observed in NB. (17,19)

b. Role of histone modifications and chromatin remodeling in gene expression in neuroblastoma

Chromatin structure and organization dictate gene activity throughout the genome. Posttranslational modifications of histone tails determine the chromatin configuration, enabling it to attain a more relaxed or condensed structure, which in turn allows for genes to become activated or silenced, respectively.(20) Acetylation of lysine residues in the histones is mediated by histone acetyltransferases (HATs) and is generally associated with active gene transcription. Current evidence regarding the role of HATs in NB is limited, however, use of the synthetic compound BF1, a HAT inhibitor, in NB cell lines caused hypoacetylation of histone 3 (H3) and of lysine (K) 18 on H3 (H3K18), which was also marked by reduced cell growth (21). Histone acetylation is not a static entity, and as such the effects of HATs are antagonized by the catalytic activity of histone deacetylases (HDACs) (22). In NB, the role of HDACs has been described by shedding light on the potential mechanisms involved in NB tumorigenesis, serving as potential markers aiding in patient stratification, as well as selection of novel therapeutic options in addition to the conventional treatment therapies. Studies showed that the following HDACs: HDAC2, SIRT1, and SIRT2 were upregulated by MYCN, which in turn promote stability and expression of MYCN protein, thus describing a positive feedback loop. (23-24)

7. Recent therapeutic modalities of neuroblastoma

a. Immunotherapy strategies

Evolution of therapeutic options for NB led to the addition of immunotherapeutic modalities to the previously recruited chemotherapeutic options. Molecular studies of the NB cells resulted in the discovery of many tumor-associated genes and antigens such as MYCN gene and GD2. MYCN gene and GD2 surface antigen are two of the most practical discoveries regarding immunotherapy of neuroblastoma. The GD2 antigen has been targeted in many animal and human studies including Phase III clinical trials. Even though these antigens have changed the face of pediatric neuroblastoma, they do not take as much credit in immunotherapy of adult-onset neuroblastoma. Monoclonal antibodies have been designed to detect this antigen on the surface of NB tumor cells. Despite bettering the outcomes for NB patients, current therapies still fail in many cases. Studies are underway to discover more specific tumor-associated antigens and more effective treatment options(25).

GD2 has been the most important antigen targeted for immunotherapy of NB(26)
.

Targeting GD2 by high specificity and high- affinity monoclonal antibodies has become a strategy for NB diagnosis and treatment (15).

ALK

NB has abnormal anaplastic lymphoma kinase (ALK) expression. ALK is ranked 33rd among NCI cancer antigens, and the majority of NBs (22 of 24 NBs) as well as half of 29 neural cell lines were found to express ALK transcripts and ALK protein. Immunodominant ALK peptide epitopes for both class I and class II major histocompatibility complex (MHC) and circulating ALK-specific T cells have been identified in ALCL patients, laying the groundwork for ALK-driven tumour immunotherapy with peptide vaccines. ProTECT (Prediction of T-Cell Epitopes for Cancer Therapy) analyses have identified two neoepitopes created by the R1275Q mutation in the ALK protein that could interact with HLA-B*15:01 to drive cytotoxic T-cell responses. Furthermore, CDX-0125-TEI, an antibody-drug conjugate directly targeting ALK receptor, demonstrated efficient ALK antigen binding and internalisation, as well as cytotoxicity against both ALK-wild and ALK-mutant patient-derived xenografts (PDXs). (27)

b. Stem cell therapy

To date, surgery, chemotherapy, and radiation serve as the first-line treatment against NBM in high-risk patients. However, few of these approaches have severe side effects. Recently, numerous studies have also reported that high chemotherapy doses, along with stem cell rescue, improvise event-free survival in patients.

Stem cells are a group of multipotent, undifferentiated cells that are capable of producing all cells in a particular tissue, organ, or organism. They have an endogenous self-renewal property. This property is tightly modulated for the normal homeostasis within the body. However, the failure of this process leads to carcinogenesis(29)

The current treatment strategy for high-risk neuroblastoma comprises induction chemotherapy, surgery, radiation, HDCT/ASCT as consolidation therapy, and various forms of post-consolidation therapy such as differentiation therapy with 13-CRA and immunotherapy.(30)Despite advances in these multimodal therapies, durable remission rates remain unsatisfactory with EFS rates less than 50%.(31, 32)

Dose intensification using HDCT/ASCT has contributed to the improvement of survival rates for patients with high-risk neuroblastoma.(33) However, further dose intensification of this type of chemotherapy is limited due to toxicity. In addition, there was no consensus on the optimal HDCT regimen in the setting of multiple HDCTs. Therefore, further optimization of the current treatment strategy and the addition of new tumor specific agents are needed to further improve treatment outcomes of patients with high-risk neuroblastoma.(34)

c. Epigenetics targeted therapies

- **Targeting epigenetics in NB:**

Several different agents have been evaluated to target the epigenetic landscape in cancer cells. These include DNA methyl-transferases (DNMTs), enzymes responsible for histone modifications (acetylation, methylation, and deacetylation) and chromatin readers.(32)

- **DNMTs:**

The expression of DNMTs is altered in NB.(33).Among the different DNMTs, DNMT3A/B expression is higher in High-risk NBs, and these enzymes are overexpressed in cisplatin- resistant NB cells.(34).A DNMT inhibitor, 5-aza-deoxycytidine (5-aza or decitabine), has been tested in NB cells and found to reduce their proliferation and colony formation.(35,36) .It has also been found that 5-aza can potentiate the efficacy of currently available chemother-apeutic drugs (cisplatin, doxorubicin, and etoposide) in NB cells.(37)

Histone modifications:

Lysine methyltransferases (PKMT), a specific type of histone methyltransferase (which induce histone modifications), have been targeted in NB.(33).In particular, BIX-01294, an inhibitor of PKMT, decreases invasion and proliferation and induces apoptosis in cells NB.(38) The addition of acetyl groups to histone lysine residues is another histone modification type, and is catalyzed by histone acetyltransferases (HATs)(39). Various HAT inhibitors, such as PU139 (a HAT pan-inhibitor) and PU141 (CBP and p300 selective inhibitors), have been investigated and found to reduce the growth of NB cells under both in vitro and in vivo conditions.(40)

Chromatin readers:

Chromatin readers identify histone modifications and recruit other proteins to the modification site to initiate or inhibit transcription. These readers include bromodomain-, tudordomain-, and chromodomain-containing proteins. Among these readers, bromodomain (BRD)-containing proteins can perform several functions, such as chromatin remodeling, histone activation, and transcriptional activation.(41) The effects of BRD inhibition in NB have been evaluated using the BET inhibitor JQ1. The treatment effectively reduces the MYCN levels and cellular growth, and induces apoptosis in vitro as well as in vivo NB models.(42)

Conclusion:

Neuroblastoma is a developmental malignancy arising within the neuronal ganglia of the periph- eral sympathetic nervous system.Several genetic alterations and epigenetic changes are implicated in the development of NB.A wide range of treatment choices is available in the different stages of localized NB.

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